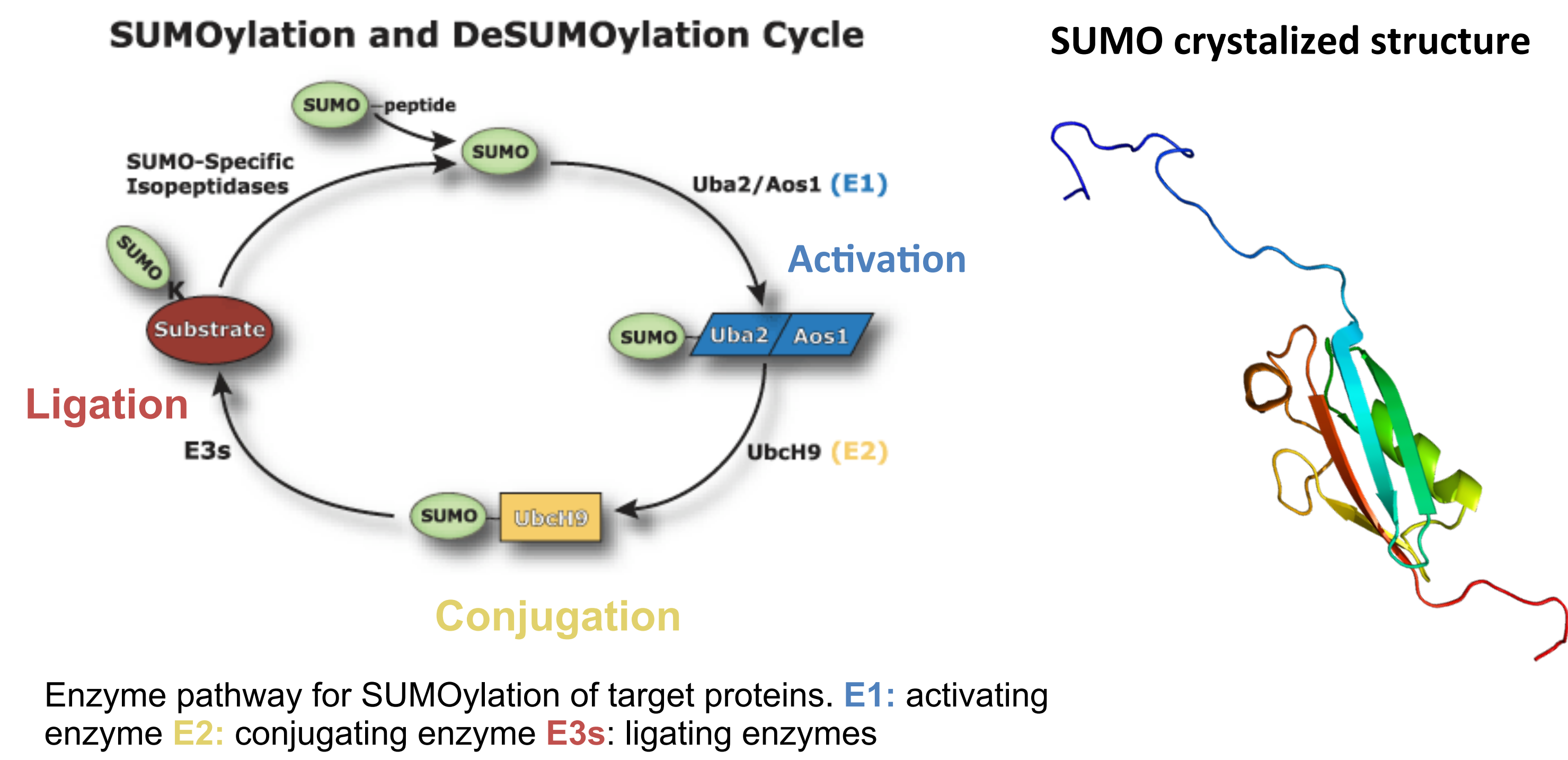


SUMOylation Status and Effects of SUMOylation on DAX-1

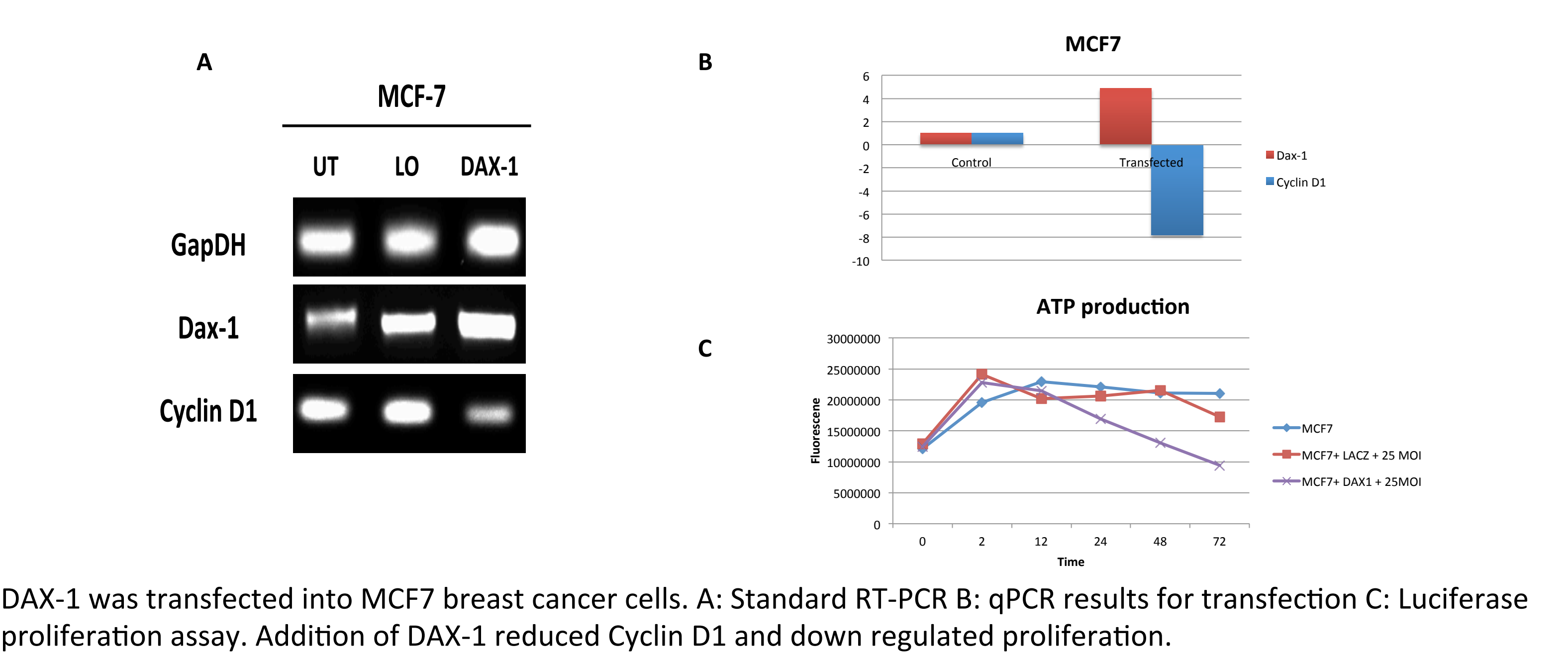
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Introduction

DAX-1 (Dosage Sensitive Sex Reversal Adrenal Hypoplasia Congenita on the X Chromosome, gene 1) is a member of the Nuclear Hormone Receptor superfamily. DAX-1 is classified as an orphan “sub-type” of nuclear receptor since, to date, no known ligand has been identified that is able to bind to the DAX-1 protein. Studies have shown that DAX-1 plays a key role early on in mammalian sex determination as well as in the expression of steroid hormones. Notably, mutation of the human DAX-1 gene results in a disorder that leads to the failure of the adrenal gland to properly develop called adrenal hypoplasia congenita (AHC). This disorder is fatal if left untreated. Furthermore, duplication or over-expression of the DAX-1 gene in humans results in individuals that are male by genotype (i.e. have X and Y chromosomes), but are phenotypically female. This is a phenotype known as dosage sensitive sex-reversal (DSS). More recently, DAX-1 has been shown to play a role in regulating growth of cancer cells, however the precise molecular mechanism of DAX-1 control is not well characterized. For example, in some types of cancer DAX-1 expression is upregulated, while in others it’s expression is highly downregulated or completely absent. In an effort to better understand DAX-1 function both in normal and disease states we are examining one type of posttranslational modification, SUMOylation. SUMOylation involves the addition of the small polypeptide conjugate SUMO (Small Ubiquitin-like Modifier) to proteins. SUMO is similar to Ubiquitin in that it is a small polypeptide, which can be covalently linked to a target protein by an isopeptide bond and that bond formation requires an enzyme pathway. However, they differ greatly in their functions; ubiquitin tags proteins for degradation whereas SUMO can have a variety of effects including changes in localization, protein-protein interaction, interaction with DNA, and in some cases stabilization of the target protein. SUMOylation of nuclear hormone receptors can have profound effects on their function. To study the effects of SUMOylation on DAX-1, the overall SUMOylation status of DAX-1 in mammalian cell lines was determined. It was found that DAX-1 is SUMOylated in several cell lines, both normal and carcinoma cells. Mutations were made in predicted SUMOylation sites within the DAX-1 gene that were identified using the SUMOsp 2.0 program program. Mutants were transfected into mammalian cell lines and assayed for changes in gene expression and activity. Here, we present the results of these experiments.



DAX-1 Down Regulates Cell Proliferation

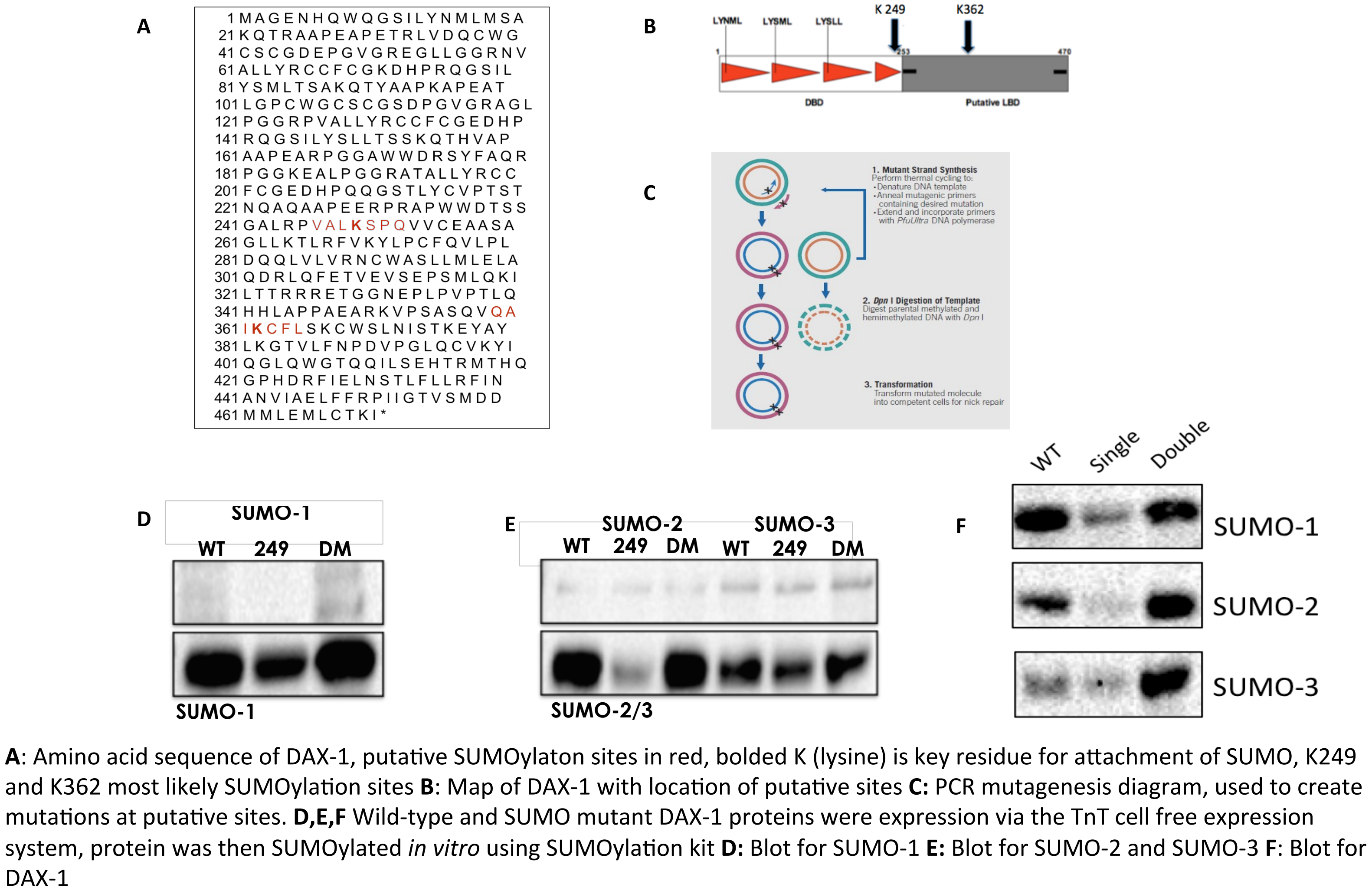


DAX-1 was transfected into MCF7 breast cancer cells. **A**: Standard RT-PCR **B**: qPCR results for transfection **C**: Luciferase proliferation assay. Addition of DAX-1 reduced Cyclin D1 and down regulated proliferation.

Methods and Materials

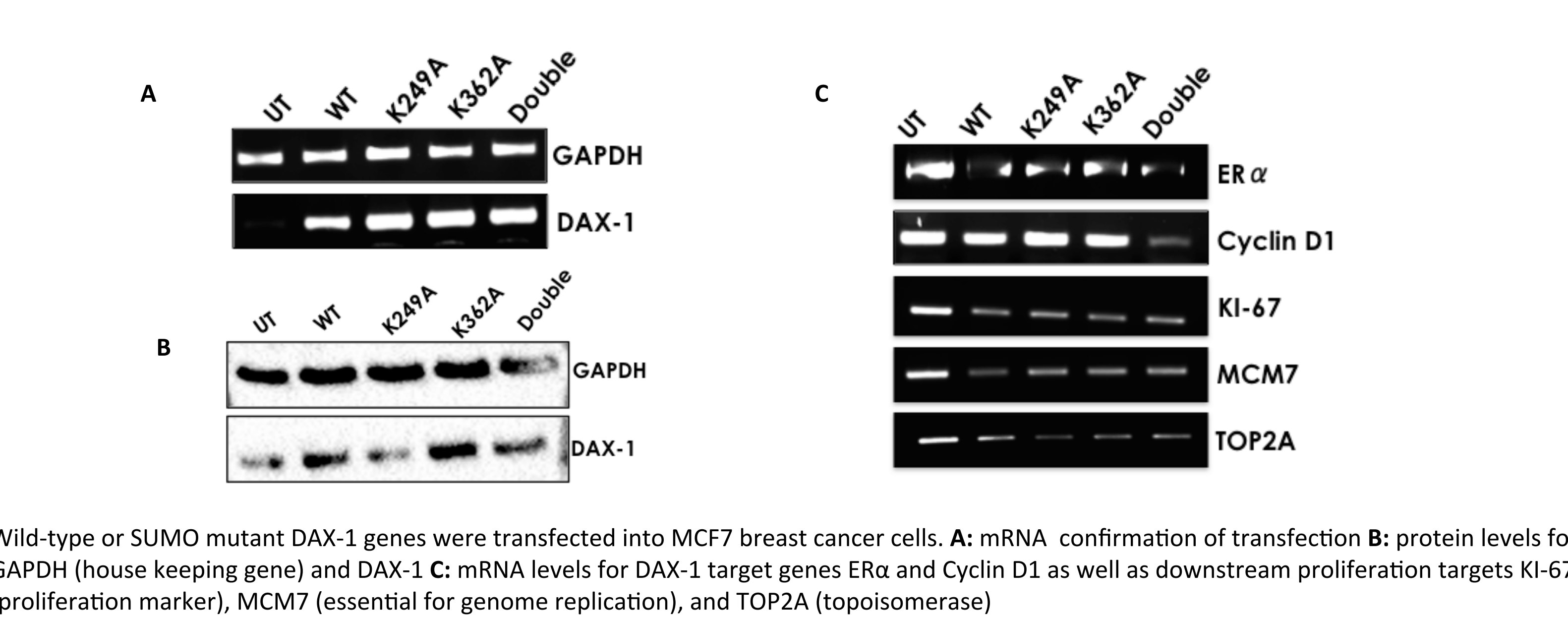
- Co-IP**
 - Use DAX-1 antibody attached to magnetic beads to pull down DAX-1 protein from whole cell lysate.
 - Follow with SDS-PAGE electrophoresis and Western Blot analysis to determine presence of SUMOylation on DAX-1 protein.
- Mutagenesis**
 - Use PCR mutagenesis of key lysine (K) amino acid residues in DAX-1 gene sequence using Stratagene QuickChange Lightning Site-Directed Mutagenesis kit. Lysine residues were changed to alanine.
- Analyze Mutants via *in vitro* SUMOylation**
 - Express wild-type and mutant DAX-1 protein using cell-free expression via TnT Quick Coupled Transcription/Translation System.
 - Carryout *in vitro* SUMOylation using ENZO SUMOylation Kit.
 - Follow up with SDS-PAGE electrophoresis and Western Blot analysis to determine SUMOylation status of mutants.
- Transfection of mutants and Assay of gene expression changes**
 - Transfect wild-type and mutant DAX-1 into cell lines.
 - Assay presence of DAX-1 using mRNA and protein.
 - Explore expression profile of wild-type and mutants.

Mutation of DAX-1 SUMOylation Putative Sites



A: Amino acid sequence of DAX-1, putative SUMOylation sites in red, bolded K (lysine) is key residue for attachment of SUMO, K249 and K362 most likely SUMOylation sites **B**: Map of DAX-1 with location of putative sites **C**: PCR mutagenesis diagram, used to create mutations at putative sites. **D, E, F** Wild-type and SUMO mutant DAX-1 proteins were expression via the TnT cell free expression system, protein was then SUMOylated *in vitro* using SUMOylation kit **D**: Blot for SUMO-1 **E**: Blot for SUMO-2 and SUMO-3 **F**: Blot for DAX-1

Expression Profile of DAX-1 Target Genes



Discussion and Conclusions

The DAX-1 protein was SUMOylated for all cell lines tested. When the putative SUMO sites were mutated, the mutant DAX-1 protein expressed, and SUMOylated *in vitro* a reduction in SUMOylation level and an overall reduction in DAX-1 level was observed.

When the SUMO mutant DAX-1 plasmids were transfected into cells with low endogenous DAX-1 levels we saw differences between the WT and mutant samples. WT DAX-1 reduced ERα and Cyclin D1 levels, some of that reduction was lost with the mutants indicating that without SUMOylation on DAX-1, DAX-1 is less able to carryout normal functions. We saw a similar result with downstream proliferation genes.

SUMOylation may be an important modification that acts as a stabilizing factor, which allows DAX-1 to function properly in the cell.

Dax-1 SUMOylation Status In Mammalian Cell Lines

